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A Thioredoxin Gene Fusion Expression System That Circumvents Inclusion Body Formation in the *E. coli* Cytoplasm

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We have developed a versatile *Escherichia coli* expression system based on the use of *E. coli* thioredoxin (*trx*A) as a gene fusion partner. The broad utility of the system is illustrated by the production of a variety of mammalian cytokines and growth factors as thioredoxin fusion proteins. Although many of these cytokines previously have been produced in *E. coli* as insoluble aggregates or "inclusion bodies", we show here that as thioredoxin fusions they can be made in soluble forms that are biologically active. In general we find that linkage to thioredoxin dramatically increases the solubility of heterologous proteins synthesized in the *E. coli* cytoplasm, and that thioredoxin fusion proteins usually accumulate to high levels. Two additional properties of *E. coli* thioredoxin, its ability to be specifically released from the *E. coli* cytoplasm by osmotic shock or freeze/thaw treatments and its intrinsic thermal stability, are retained by some fusions and provide convenient purification steps. We also find that the active-site loop of *E. coli* thioredoxin can be used as a general site for small peptide insertions, allowing for the high level production of soluble peptides in the *E. coli* cytoplasm.

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The production of protein pharmaceuticals in *Escherichia coli* is a cornerstone of the biotechnology industry. However a number of difficulties are frequently encountered when expressing heterologous genes in this organism. For example, the significant differences that exist between *E. coli* and human genes, both in their patterns of codon usage and in their translation initiation signals, can interfere with the efficient translation of human messenger RNA on bacterial ribosomes[1]. Alternatively, heterologous proteins synthesized in *E. coli* may fail to accumulate to significant levels due to the activity of host cell proteases[2]. In addition, the physical characteristics of many therapeutically useful proteins can cause problems, since many in their native state are secreted molecules requiring glycosylation and disulfide-crosslinking for both stability and solubility. Such stabilizing influences are unavailable in the bacterial cytoplasm, with the result that heterologous proteins made within *E. coli* often appear as insoluble aggregates known as "inclusion bodies"[3,4].

Although production of a protein as an insoluble "inclusion body" can offer the advantage of an easy purification, devising an appropriate solubilization and refolding procedure is an empirical process, with no guarantee of success in all cases. As an alternative to refolding misfolded proteins produced in the *E. coli* cytoplasm, the secretion of proteins into the periplasm of *E. coli* has sometimes proven successful[5,6]. However the yields of protein obtained from these *E. coli* secretion systems rarely approach those obtained by intracellular expression, and in many instances problems with protein stability and solubility in the periplasm have been reported[7,8].

A popular strategy that avoids some of the problems associated with other expression methods is to link the gene of interest to a second gene which is already known to be expressed well in *E. coli*, to generate a fusion protein. Most of the successful fusion protein systems position the protein of interest at the C-terminal end of the highly-expressed fusion partner, to ensure efficient translation initiation. However although some of the original fusion systems, such as those employing *E. coli lacZ*[9] or *trp*E[10] as the highly-expressed partners, can successfully resolve translation initiation difficulties, they do not always solve the intrinsic solubility problems of heterologous proteins made in *E. coli*. The fusion proteins produced by these systems are also frequently found in inclusion bodies. There are other systems, such as those employing *Staphylococcus* protein A[11], *Schistosoma* glutathione-S-transferase[12], and the *E. coli* maltose binding protein, *mal*E[13], as fusion partners, that are much more successful in producing soluble fusion proteins. Additionally in each of these cases the fusion partner provides a distinct biochemical property, for example amylose binding in the maltose-binding protein system, which can be exploited as an affinity tag for fusion protein purification.

Here we describe a new fusion gene expression system based on the use of *E. coli* thioredoxin (*trx*A)[14] as the fusion partner. We have found that *E. coli* thioredoxin bears a number of characteristics which make it a particularly suitable choice in this role. When over-expressed from plasmid vectors, *E. coli* thioredoxin can accumulate to 40% of the total cellular protein[15], and even at these expression levels all of the protein remains in the soluble fraction. Since thioredoxin is small (11,675 kD) it usually represents a relatively modest portion of any fusion protein, in contrast to other systems where the fusion partner itself may comprise most of the fusion's total mass. The tertiary structure reveals that both the N- and C- termini of thioredoxin are accessible on the molecule's surface[16], in good positions for potential fusions to other proteins. We show here that a wide variety of secreted mammalian cytokines and growth factors can be successfully produced in a soluble form in the *E. coli* cytoplasm as C-terminal fusions to thioredoxin. The tertiary structure also shows that the characteristic thioredoxin active-site, -CGPC-, protrudes from the body of the protein as a surface loop. We find that this thioredoxin active-site loop can be used as a site for internal peptide fusions.

Thioredoxin possesses two further characteristics which can be exploited for fusion protein purifications. The first is the inherent thermal stability of the molecule, a property that is retained by some thioredoxin fusions and which enables heat treatments to be used as an effective purification tool. The second additional property relates to thioredoxin's cellular loca-

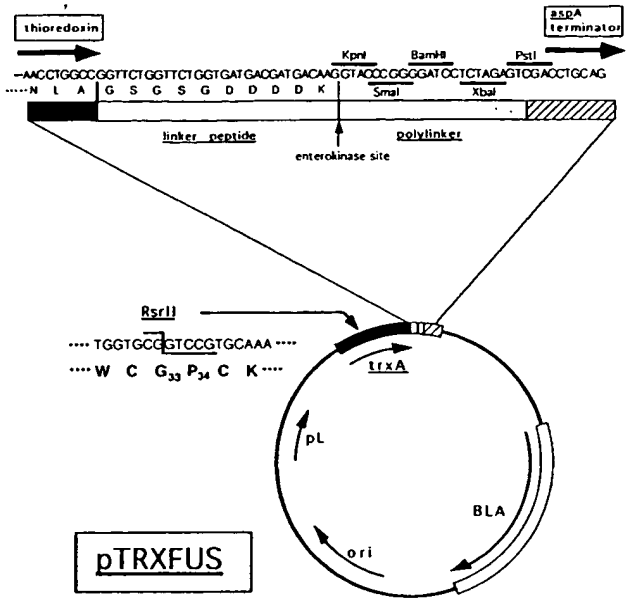

FIGURE 1. A diagram illustrating the main features of the thioredoxin gene fusion expression vector, pTRXFUS. The DNA sequence is shown for the 3′-end of the thioredoxin gene, the "linker" region encoding an enterokinase (enteropeptidase) cleavage site, and a "polylinker" sequence containing convenient restriction endonuclease cloning sites. The sequence surrounding the active-site loop of thioredoxin is also shown to illustrate the single RsrII site which can be used for peptide sequence insertions at this location. Abbreviations used are: *trx*A, the *E. coli* thioredoxin gene; BLA, (β-lactamase gene; ori, colE1 replication origin; pL, bacteriophage λ major leftward promoter; *asp*A terminator, *E. coli* aspartate aminotransferase transcription terminator.

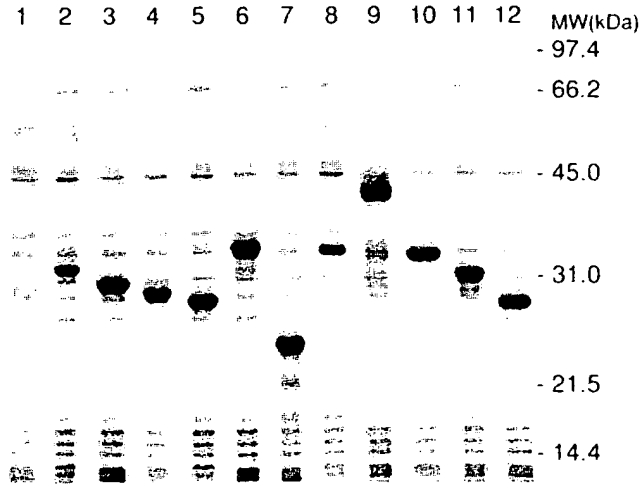

FIGURE 2. The proteins found in the *soluble* fractions derived from *E. coli* cells expressing eleven different thioredoxin gene fusions. The numbers in parentheses refer to the growth temperature chosen for expressing each particular fusion. Lane 1: host *E. coli* strain GI724 (negative control, 37°C), lane 2: murine IL-2 (15°C), lane 3: human IL-3 (15°C), lane 4: murine IL-4 (15°C), lane 5: murine IL-5 (15°C), lane 6: human IL-6 (25°C), lane 7: human MIP-1α (37°C), lane 8: human IL-11 (37°C), lane 9: human M-CSF (37°C), lane 10: murine LIF (25°C), lane 11: murine SF (37°C), and lane 12: human BMP-2 (25°C). Shown is a 10% SDS-PAGE gel, stained with Coomassie blue.

tion. Although *E. coli* thioredoxin is a cytoplasmic protein, it has been shown to occupy a special position within the cell, being mainly located on the cytoplasmic face of the adhesion zones that exist between the inner and outer membranes of the *E. coli* cell envelope[17]. From this location thioredoxin has been shown to be quantitatively released to the exterior of the cell by simple osmotic shock or freeze/thaw treatments[18], a remarkable property which we show here is retained by some thioredoxin fusion proteins, providing a simple and convenient purification step.

Results

Production of fusion proteins. The *E. coli* expression vector pTRXFUS (Fig. 1) was used as the basis for joining genes encoding a variety of mammalian growth factors and cytokines to the 3′- end of *E. coli trx*A. The coding sequence for each cytokine was inserted into pTRXFUS at the unique KpnI site in the 3′-polylinker to create an in-frame translational fusion. Gene expression under the transcriptional control of the bacteriophage lambda pL promoter was performed in strain GI724, with the growth temperature for expression in each case chosen so as to maximize soluble accumulation of the particular fusion protein, ranging from 15°C to 37°C.

To assess the expression and solubility of each of the fusion proteins, cultures were harvested approximately four hours post-induction, the cell pellets were lysed in a French pressure cell and the resulting lysates separated into soluble and insoluble fractions by centrifugation at 15,000xg (see Experimental Protocol). The soluble portion was loaded onto a 10% SDS-polyacrylamide gel. Figure 2 shows that each of the fusion proteins was expressed well, to approximately 5–20% of the total cell protein. Importantly, all of the fusion proteins were present mainly or entirely in the *soluble* cellular fraction under the growth conditions chosen. In contrast, previous attempts at intracellular expression of many of these proteins in *E. coli* have been reported by other groups, and have invariably led to the formation of insoluble "inclusion bodies", for example with: IL-2 (ref. 19), IL-3 (ref. 20), IL-4 (ref. 21), IL-6 (ref. 22), SF (ref. 23), and M-CSF (ref. 24). In fact, we have observed that IL-3, IL-6, and BMP-2 are produced in inclusion bodies at 15°C when expressed using a comparable vector and strain but without the aid of fusion to thioredoxin (data not shown).

Many of the clarified lysates containing soluble fusion proteins were tested for *in vitro* biological activity (Table 1). All of the fusions which contained heterologous partner proteins usually found as monomers in their natural state exhibited some biological activity. A number of the fusions tested, including the the IL-3, IL-6 and IL-11 fusions, were fully active. Others, such as the IL-2, IL-4, IL-5, LIF and SF fusions, displayed significant activity, sometimes diminished when compared to the native factors. Partial biological activity would not be unexpected for a subset of thioredoxin-cytokine fusion proteins, since for some the receptor binding site may be obscured by the thioredoxin domain. Thioredoxin fusions to covalently-linked dimeric heterologous proteins, for example BMP-2 and M-CSF, were inactive in bioassays, perhaps due to their inability to form dimers when fused to thioredoxin. However, MIP1α, while inactive as a thioredoxin fusion, attained full bioactivity when cleaved from its partner (data not shown).

The high levels of solubility and biological activity observed in most cases suggests that heterologous proteins can, in general, assume their proper native conformations while fused to thioredoxin and produced in the *E. coli* cytoplasm.

Thermal stability of thioredoxin fusions. A remarkable characteristic of *E. coli* thioredoxin is its ability to withstand prolonged incubation at elevated temperatures without undergoing irreversible thermal denaturation. Indeed, purification procedures for isolating *E. coli* thioredoxin from cell lysates have utilized 85°C incubations for precipitating most of the other proteins, while retaining thioredoxin in the soluble fraction[14]. We find that this property of thermal stability is shared by some

thioredoxin fusion proteins, providing in those cases a simple and rapid purification step. Figure 3 shows the time course for an 80°C heat-treatment of a cell lysate from a strain engineered to express a thioredoxin/MIP-1α fusion protein. The fusion remained fully soluble throughout the 10 minute incubation period at 80°C. Most contaminating *E. coli* proteins denatured and precipitated after just one minute, resulting in a 5-fold purification of the fusion protein in the recovered soluble fraction. Since this kind of purification step is dependent on the thermal stability of each particular heterologous partner protein, it will not be applicable to all thioredoxin fusions. However it is a useful option of the thioredoxin system that is unavailable with other fusion systems. Additionally we have observed that peptide insertions into the active-site loop of thioredoxin (see below) also often retain the thermal stability of native thioredoxin, affording easy purifications for these molecules.

Selective release of thioredoxin fusions. *E. coli* thioredoxin has been shown to preferentially reside at sites around the inner periphery of the cytoplasmic membrane in *E. coli* known as adhesion zones, or Bayer's patches[17,25]. At these sites there are gaps in the peptidoglycan cell wall where the inner and outer cell membranes are fused together, leading to an adjacent "osmotically sensitive" cellular compartment whose contents can be released to the surrounding medium by a rapid osmotic shock treatments in the presence of EDTA. It has been observed, perhaps surprisingly, that two particular *E. coli* cytoplasmic proteins, EF-Tu and thioredoxin, can be selectively released by such treatment. Indeed thioredoxin has been reported to be *quantitatively* expelled from the cytoplasm by osmotic-shock treatments, while most other cytoplasmic proteins remain encapsulated by the cytoplasmic membrane[18].

To test whether thioredoxin fusion proteins would also display selective localization and release, we subjected cells expressing different thioredoxin/cytokine fusion constructs to osmotic-shock treatments (see Experimental Protocol). Figure 4 shows the results obtained with overexpressed *E. coli* thioredoxin, the MIP-1α fusion and the IL-11 fusion. As reported previously[18] thioredoxin itself was quantitatively released by osmotic shock, even when produced at >20% of the total cell protein (Fig. 4, lane 2). Cells expressing thioredoxin/MIP-1α were found to release about 30% of the fusion protein upon osmotic shock (Fig. 4, lane 5). Up to 70% of the thioredoxin/IL-11 fusion could be osmotically released from the cytoplasm, resulting in a significant purification (Fig. 4, lane 8) for a non-

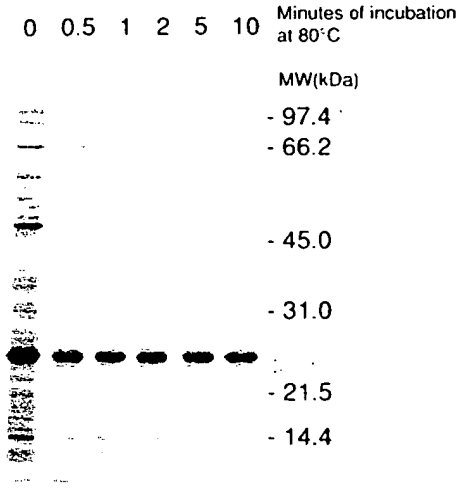

FIGURE 3. Protein remaining in solution following heat-treatment of a thioredoxin fusion protein at 80°C. *E. coli* cells producing the thioredoxin/MIP-1α fusion protein were lysed in a french pressure cell and the lysate heated at 80°C. Aliquots were removed at the specified times, cooled on ice, clarified by centrifugation, and the soluble fractions loaded onto a 10% SDS-PAGE gel. Proteins were visualized with Coomassie blue.

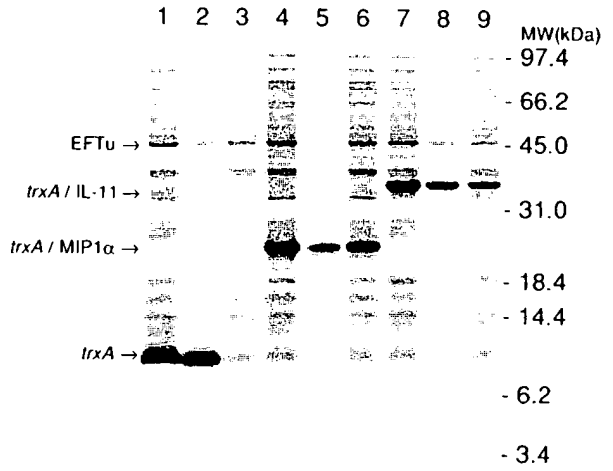

FIGURE 4. Selective osmotic release of thioredoxin fusion proteins. *E. coli* cells producing native thioredoxin (lanes 1–3), the thioredoxin/MIP-1α fusion (lanes 4–6), and the thioredoxin/IL-11 fusion (lanes 7–9) were subjected to an osmotic release procedure (see Experimental Protocol). Samples representing equivalent amounts of whole cells (lanes 1,4,7), material released from whole cells by osmotic shock (lanes 2,5,8), and residual proteins not released from cells by osmotic shock (lanes 3,6,9), were run on a 10% tricine SDS-polyacrylamide gel and were subsequently visualized with Coomassie blue. The mobilities on this gel of thioredoxin, the thioredoxin/MIP-1α and thioredoxin/IL-11 fusion proteins, and EFTu are shown by arrows.

TABLE 1. *In vitro* biological activities, in crude bacterial lysates, for various thioredoxin/cytokine fusion proteins.

Thioredoxin Fusion	Specific Activity (units/mg)[a]	Percent Activity of Native Cytokine[b]	Assay Name	Reference
murine IL-2	1.1×10^5	n/d	32D clone 23	[59]
human IL-3	5.5×10^6	100	M-07e	[57]
murine IL-4	1.7×10^5	n/d	32D clone 23	[59]
murine IL-5	1.8×10^5	n/d	TF-1	[61]
human IL-6	4.8×10^6	100	T1165	[27]
human MIP1α	not active[c]	0	CFU-A	[60]
human IL-11	2.5×10^6	100	T1165	[27]
human M-CSF	not active	0	bone marrow	[62]
murine LIF	2.5×10^6	2.5	DA1a	[63]
murine SF	5.0×10^3	n/d	M-07e	[57]
human BMP-2	not active	0	W-20	[58]

[a]Specific activity is expressed as dilution units per milligram of cytokine. Activities were measured in crude bacterial lysates, the protein concentrations of fusions present in these lysates were estimated from stained SDS-polyacrylamide gels. [b]In a number of cases the activity of a native cytokine in a particular assay was not known. In these instances comparisons with the activities of thioredoxin fusions could not be made (signified by n/d). [c]Although the thioredoxin-MIP1α fusion protein was itself inactive, fusion-derived MIP1α was fully active in the CFU-A assay.

chromatographic procedure. It is interesting to notice in Figure 4 that approximately half of the EF-Tu present in these cells was also released by the osmotic shock treatments.

As an alternative means of selective release, equivalent results were obtained when cells were subjected to a simple freeze/thaw treatment in the presence of EDTA, instead of the

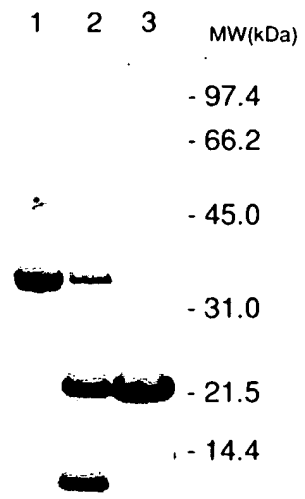

FIGURE 5. Specific cleavage of the thioredoxin/IL-11 fusion protein by enteropeptidase. Shown is a Coomassie blue-stained, 10% tricine SDS-polyacrylamide gel of: partially-purified thioredoxin/IL-11 fusion protein (lane 1), the same protein sample following cleavage with enteropeptidase (lane 2), and IL-11 subsequently purified from the reaction products (lane 3).

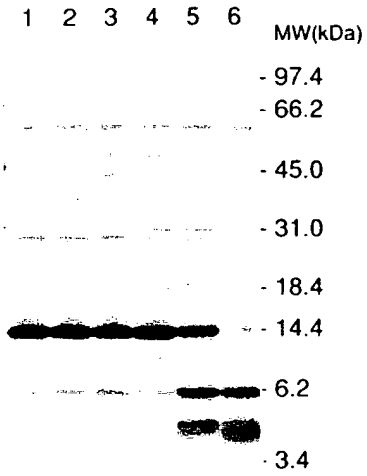

FIGURE 6. Specific cleavage of TRX-MPHANT by enteropeptidase. Partially purified TRX-MPHANT, a thioredoxin fusion protein bearing a 25 residue insertion in the active site loop, was incubated in a reaction with (lanes 4–6) or without enteropeptidase (lanes 1–3). Reaction products were run on a 10% tricine SDS-polyacrylamide gel, which was subsequently stained with Coomassie blue. Shown are timepoints taken at the start of the incubation (lanes 1 and 4), after 5h (lanes 2 and 5), and after 22h (lanes 3 and 6).

osmotic shock procedure (data not shown). This had previously been reported for native thioredoxin[18] and was found to work well for some fusions. The yields of released material following the selective release procedures varied depending upon the particular C-terminal fusion partner protein, and on the growth stage of the cells (J.M.M. and E.A.D., unpublished data). Release was at a maximum during exponential growth, and decreased as cells approached stationary phase.

Cleavage of thioredoxin fusion proteins. The ability to efficiently cleave and separate thioredoxin from fused polypeptides is essential for this expression system to be a practical method for producing therapeutically useful proteins in *E. coli*. With this in mind we designed the linker peptide lying between the thioredoxin and fused C-terminal domains to include the sequence -DDDDK-, which is the recognition sequence for the mammalian intestinal protease enteropeptidase. Enteropeptidase cleaves specifically following the P_1 lysine residue[26], and is tolerant to a wide variety of amino-acid residues in the P_1' position (E.R.L. and L.A.C., unpublished observations). A thioredoxin/human IL-11 fusion protein was produced and purified from *E. coli* cell lysates, as described in the Experimental Protocol, and incubated in a reaction with bovine enteropeptidase. Figure 5 shows that the thioredoxin/IL-11 fusion in this reaction was cleaved specifically at the enteropeptidase cleavage site in the linker peptide, resulting in a 21 kD product corresponding to mature human IL-11, and a 13 kD product consisting of thioredoxin with the 10 amino acid linker peptide still attached to its C-terminus (lane 2). Amino-terminal sequencing of the IL-11 product confirmed that cleavage was specific at the expected site, leaving a homogeneous N-terminus (data not shown). Under the reaction conditions most of the fusion protein was cleaved in 15 hours. The cleavage could be driven to completion, however, by longer incubation or by the addition of more enzyme. Separation of the cleaved IL-11 product from both the thioredoxin/linker portion and the remaining uncleaved fusion protein was easily achieved by exploiting the differences in charge between the three components. Mature human IL-11 is very basic, with a pI (predicted from its amino acid sequence) of 11.7[27]. *E. coli* thioredoxin is acidic, with a pI of about 4.5[14], with the four aspartic acid residues in the linker peptide increasing this predominance of negative charge. As a result, IL-11 was efficiently separated from the other components of the cleavage reaction by chromatography on QAE-Toyopearl. At pH 8.0 in a low ionic strength buffer (25 mM HEPES), cleaved IL-11 did not bind anion-exchange resins and was recovered at 100% yield (Fig. 5, lane 3). Uncleaved fusion and the thioredoxin/linker moiety bound quantitatively and were effectively removed. Bovine enterokinase also bound to anion exchange resins and was quantitatively removed from the IL-11 product. In addition, a substantial reduction in endotoxin levels was achieved in this step (data not shown). The resulting IL-11 exhibited full bioactivity in the T1165 assay[27].

Expression of peptides in the thioredoxin active-site loop. Often it may be desirable to produce short peptide sequences in *E. coli*, for instance to use as immunogens or for screening purposes[28]. However, such peptides frequently have inherent solubility problems, or they may be sensitive to degradation by host cell proteases. As a way to avoid these problems we tested thioredoxin's suitability as a general fusion partner for peptide expression in *E. coli*.

We suspected that peptides fused at the N- or C-termini of thioredoxin might be susceptible to *E. coli* amino- and carboxypeptidases. That suspicion was confirmed by the low expression level and proteolytic lability we observed when we attempted to produce a thioredoxin fusion protein with a 20-residue C-terminal peptide extension (data not shown). To avoid this potential problem, we chose instead to use an internal location within thioredoxin as a peptide fusion site. The tertiary structure of thioredoxin shows that the active-site of the molecule, -C_{32}-G-P-C_{35}-, is a surface loop that protrudes from the body of the protein, and thus presumably contributes little to overall structural stability. A convenient RsrII restriction site lies in the DNA sequence encoding the loop, cutting between G_{33} and P_{34}. This RsrII site was used to introduce a segment of DNA encoding a 25 residue peptide sequence, -PGSGRPLAVKVFSYIDDDDK GPGSG-, into the thioredoxin gene. The resulting fusion protein (called TRX-MPHANT) was found to accumulate to high levels in *E. coli*, and was equally as stable to 80°C heat treatments as wild-type thioredoxin (data not shown), indicating that a peptide

insertion into the active site loop did not adversely effect the folding or final stability of the thioredoxin/peptide fusion molecule. We have inserted a wide variety of other peptide sequences of between 14 and 25 residues in length at this location and have found that the active-site loop is very permissive, almost invariably allowing for the high-level stable expression of most peptides as fusions. The great majority of these fusion proteins are soluble.

By design the peptide insert in TRX-MPHANT contains an enteropeptidase recognition sequence, -DDDDK-. To determine whether or not this insert was displayed on the surface of thioredoxin, partially purified TRX-MPHANT was incubated in the presence of enteropeptidase (Fig. 6, lanes 4–6). A specific cleavage was observed which was not seen when enteropeptidase was either omitted from the reaction (Fig. 6, lanes 1-3), or when native thioredoxin was used in place of the TRX-MPHANT fusion (data not shown). The specific cleavage by enteropeptidase of TRX-MPHANT indicates that the inserted peptide in this fusion is in an accessible location in thioredoxin. In further support of this notion we have raised monoclonal antibodies, using TRX-MPHANT as the immunogen, which specifically bind to TRX-MPHANT itself but which do not bind to native thioredoxin. This further demonstrates that peptides inserted into the active-site loop of thioredoxin are accessible, and that such fusions are potentially useful immunogens (data not shown).

Discussion

In contrast to many other gene fusion systems, the thioredoxin gene fusion system provides a solution for a major problem which has bedeviled heterologous gene expression in *E. coli*: the formation of inclusion bodies. We have demonstrated the utility of the system by producing a total of eleven different mammalian cytokines and growth factors as soluble, C-terminal, thioredoxin fusions in the *E. coli* cytoplasm. For these proteins the thioredoxin fusion system has obviated the time-consuming process of *in vitro* protein refolding, since most have been produced previously in *E. coli* only in an insoluble form. Not only are these proteins soluble as thioredoxin fusions, but many also exhibit high levels of bioactivity—indicating that they have adopted their correct conformations. In addition, there are three other advantages that the thioredoxin fusion system can provide: high overall gene expression levels, a simple initial purification step through a selective release of fusion protein from the *E. coli* cytoplasm by osmotic-shock, and the ability to use heat treatments as an easy purification tool.

We note that the use of thioredoxin as a fusion partner has several naturally occurring counterparts. For example, the sequences of the mammalian protein disulfide isomerases (PDI) can each be thought of as comprising two complete "thioredoxin domains" flanking a central "non-thioredoxin domain" of 200 amino acids[29]. An identical arrangement of terminal full length "thioredoxin domains" has been identified in the sequence of rat phosphoinositide-specific phospholipase C (PLC-I)[30], although PDI and PLC-I share no other structural or functional homologies in the central "non-thioredoxin" region. A third instance of a naturally occurring thioredoxin fusion protein is a unique arrangement of three complete "thioredoxin domains", two at the N-terminus and one at the C-terminus, flanking a central 200 residue long "non-thioredoxin" region in mammalian ERp72[31].

The vast majority of thioredoxin fusion proteins that we have produced are well expressed and produce protein products that are both stable in the *E. coli* cytoplasm and exhibit the expected biological activity of the fused heterologous protein. A subset of these fusion proteins are selectively released by osmotic shock, and some are resistant to thermal denaturation. These properties all suggest that both thioredoxin and the fused heterologous protein are able to fold correctly when linked together. Since thioredoxin has a very tight tertiary fold, with over 80% of the polypeptide chain involved in elements of strong secondary structure, its folding pathway is probably very resistant to any perturbations that may be caused by the presence of fused heterologous proteins. This may in fact be the underlying reason that explains why thioredoxin is such a good fusion partner. Thioredoxin's robust folding characteristics are further illustrated by our finding that fusions carrying peptide insertions in the active site-loop not only fold normally, but often retain the inherent thermal stability of the wild-type protein (e.g. TRX-MPHANT).

Why should heterologous proteins fold correctly in the *E. coli* cytoplasm as fusions to thioredoxin when by themselves they would accumulate in inclusion bodies? It has been suggested that inclusion bodies arise by the inappropriate aggregation of partially-folded or incorrectly-folded intermediates[3]. It is possible that by physically linking a heterologous protein to a stable and highly soluble fusion partner such as thioredoxin these aggregates might be prevented from forming, allowing correct folding to occur eventually. The fact that thioredoxin is translated first may allow it to fold before its nascent C-terminal fusion partner, in this way being able to passively "interact" with the partner as it emerges from the ribosome. The nature of this "interaction" is unclear, one possibility is that thioredoxin would act as a covalently-linked "molecular chaperon", fulfilling a solubilizing role similar in some ways to authentic chaperon proteins[32]. In contrast to the *trans*-acting chaperonins, however, co-translation would *dictate* a close physical contact between the thioredoxin and heterologous partner protein "domains", facilitating any potential interactions. Indeed we have found that enhanced production of IL-1l in *E. coli* required a physical linkage to thioredoxin, and could not be accomplished by merely increasing intracellular concentrations of thioredoxin in *trans* (data not shown). It is important to note that the surface of thioredoxin has evolved to allow physical contact with a number of different proteins in its role as an intracellular oxidoreductase[14]. This property may also help to explain its ability to confer solubility on partner proteins.

Disulfide crosslinks can be important stabilizing structures for proteins[33], yet the reducing environment of the *E. coli* cytoplasm makes stable disulfide formation very difficult, leading to another possible mechanism for inclusion body formation in *E. coli*: thermal denaturation and precipitation of heterologous proteins at physiological temperatures due to an inability to form disulfides. Perhaps surprisingly we find that many mammalian proteins that are disulfide crosslinked in their natural state are expressed as soluble thioredoxin fusions at physiological temperatures in *E. coli*. Maybe thioredoxin, by acting as a "chaperon", can keep partially denatured proteins lacking their normal disulfides from forming into insoluble aggregates. We have noticed that some thioredoxin fusions become more soluble as the growth temperature of cells expressing them is lowered. This has also been reported for a number of heterologous proteins expressed alone in the *E. coli* cytoplasm[34], and may be due to a reduction in thermal denaturation. Alternatively the lower growth temperature may diminish the strength of some inappropriate hydrophobic interactions that may otherwise lead to aggregate formation. Nevertheless we find that for any particular growth temperature the thioredoxin fusion is invariably much more soluble than the heterologous protein expressed by itself.

We have also not yet eliminated the possibility that the overexpression of thioredoxin or thioredoxin fusions can change the redox environment of the *E. coli* cytoplasm, or that over-

expressed thioredoxin or thioredoxin fusions acting as protein disulfide oxido-reductases[14] can act directly on heterologous proteins.

The synthesis of short peptide sequences in *E. coli* has proven difficult in the past—they are usually either rapidly degraded by host peptidases or, less frequently, accumulate in an insoluble form. We have shown that thioredoxin contains a permissive site that allows for the insertion of a wide variety of peptide sequences. Peptides can now be made, soluble and in large amounts, as internal fusions into the thioredoxin active-site loop. Moreover in that location peptides occupy an accessible position on the protein's surface. One potential use for this is as a convenient way of generating immunogens. However a more exciting possibility is the use of thioredoxin as a vehicle for producing libraries of random peptide sequences intracellularly in *E. coli* or indeed in other organisms—since the inherent stability of *E. coli* thioredoxin should not be limited to the bacterial cytoplasm.

Osmotic release of heterologous proteins from the cytoplasm of *E. coli* is a rare attribute which has been described in just a few instances[35,36]. Thioredoxin fusions now enable this highly desirable property to be extended to other heterologous proteins. Osmotic release is a rapid, inexpensive and effective technique that not only removes the majority of protein contaminants, but also removes most of the nucleic acids that are normally present in bacterial lysates. The mechanism of the process is unclear, but the quantitative release of *E. coli* thioredoxin by osmotic shock[18] suggests efficient targeting to an osmotically-sensitive cytoplasmic compartment, probably by a specific thioredoxin sequence or some element of tertiary structure. Our finding that a number of thioredoxin fusion proteins can also be released in good yield from the cell by osmotic shock suggests that this targeting element remains effective. However we also find that not all thioredoxin fusions can be efficiently released, perhaps in these cases because the targeting element is obscured by the heterologous protein "domain".

Cleavage of the fusion protein is clearly an essential part of any fusion expression system. We chose to use a protease with high specificity and broad utility, enteropeptidase[26], as our initial cleavage reagent and have shown that it can be highly effective in that role. However the cleavage site in the linker region of our thioredoxin fusion vector can be easily exchanged for other sites as necessary, and many of the different agents, both enzymatic and chemical, that have been used previously to cleave fusion proteins[37] are applicable to the thioredoxin system. With this added flexibility, we expect that the thioredoxin gene fusion expression system will prove useful both for small-scale research applications as well as for the large-scale production of biopharmaceuticals.

Experimental Protocol

Bacterial strains and plasmids. All expression work was performed in *E. coli* K12 strain GI724, (ATCC 55151) a derivative of RB791 (ref. 38) (= W3110 lacI^q*lacP*L8), which contains the bacteriophage λ repressor (*cI*) gene stably integrated into the chromosomal *amp*C locus. The *cI* gene is under the transcriptional control of a synthetic *Salmonella typhimurium trp* promoter, integrated upstream of *cI* in *amp*C. The thioredoxin fusion expression vector, pTRXFUS (Fig. 1) was constructed using standard techniques[39]. The plasmid is based on pUC-18 (ref. 40) and contains a colE1 origin of replication, a β-lactamase gene as a selectable marker, and the bacteriophage λ pL promoter[41] located upstream of the *E. coli trx*A gene. A short DNA sequence encoding a linker peptide, "-GSGSGDDDDK-" is positioned at the 3'-end of *trx*A, serving not only to fuse heterologous proteins with thioredoxin but also providing a specific enteropeptidase site[26] to allow for subsequent cleavage. Further downstream in the vector lies a polylinker DNA sequence containing convenient restriction sites and also a transcription terminator sequence based on that found following the *E. coli asp*A gene[42]. A variety of mammalian cytokine and growth factor cDNA sequences were introduced into the pTRXFUS vector, including those encoding mature forms of human IL-3 (ref. 43), IL-6 (ref. 44), MIP-1α (ref. 45), IL-11 (ref. 27), BMP-2 (ref. 46) and M-CSF (residues 1-223) (ref. 47), and also the murine cytokines IL-2 (ref. 48), IL-4 (ref. 49), IL-5 (ref. 50), LIF (ref. 51) and steel factor (SF, residues 1-164) (ref. 52). Wild-type *E. coli* thioredoxin was expressed using a vector, pALtrxA-781, which contained the normal *trx*A translation terminator in place of the sequence encoding the 3'-linker peptide found in pTRXFUS. The DNA sequence encoding the active-site loop of *E. coli trx*A contains a site for the restriction enzyme RsrII (Fig. 1). This site is unique in pALtrxA-781, facilitating the introduction of synthetic DNA sequences at this location in thioredoxin. The plasmid pALtrxA-MPHANT codes for a thioredoxin fusion containing the 25 residue peptide sequence "-PGSGRPLAVKVFSYIDDDDKGP GSG-" inserted into the active-site loop.

Expression of fusion proteins. Strain GI724 containing the thioredoxin fusion expression plasmid of interest was grown at the desired temperature in IMC broth (M9 medium[53] supplemented with 0.5% w/v glucose, 0.2% w/v casamino acids and 100μg/ml ampicillin) until the culture reached an A_{550} of 0.5. In a manner similar to that described by Mieschendahl et al.[54], the amount of transcription initiated from the pL promoter on pTRXFUS is determined by the level of *cI* in GI724, which itself is controlled by cytoplasmic tryptophan levels. In the presence of high levels of tryptophan, *cI* synthesis is repressed in the host strain, transcription from the pL promoter is induced, and plasmid-directed gene expression proceeds. Fusion protein synthesis induced by the addition of 100μg/ml tryptophan to the culture medium was allowed to continue for a desired time, typically 4 hours. Lysates were prepared by suspending the cells in 1mM phenylmethylsulfonyl fluoride (PMSF)/1mM *p*-aminobenzamidine (PABA)/20mM Tris-Cl, pH8 and then passing them through a French pressure cell at 20,000 p.s.i. Proteins in the "soluble" fraction of these lysates were defined as those that remained in the supernatant following centrifugation at 15,000xg for 15 minutes.

Heat treatments. *E. coli* cells expressing the *trx*A/MIP-1α fusion were resuspended in 2.5mM EDTA/20mM Tris-Cl, pH8, to a concentration of 100 A_{550} units/ml before lysis in a French pressure cell at 20,000 p.s.i.. The unclarified lysate was then heated at 80°C, with aliquots removed at various times. Each aliquot was cooled quickly on ice before clarification by centrifugation at 15,000xg for 10 minutes. Soluble proteins present in the supernatant fraction were analyzed on a 10% SDS-polyacrylamide gel[55].

Osmotic shock fractionations. *E. coli* cells expressing native thioredoxin, the *trx*A/MIP-1α fusion, or the *trx*A/IL-11 fusion were resuspended in ice-cold 20% sucrose/2.5mM EDTA/20mMTris-Cl, pH8, to a concentration of 5A_{550} units/ml and kept on ice for 10 minutes. The cells were then pelleted by brief centrifugation at 15,000xg for 30 seconds before being resuspended gently in an equivalent volume of ice-cold 2.5mM EDTA/20mMTris-Cl, pH8. After a second 10 minute incubation on ice, the cells were again pelleted by centrifugation at 15,000xg, this time for 10 minutes. Samples of the original cells, the final supernatant ("shockate"), and pellet fractions were analyzed on a 10% SDS-polyacrylamide gel[56].

Purifications. All purification steps were performed at 4°C. Cells expressing the *trx*A/IL-11 fusion were resuspended in 25mM Hepes, pH 8.0/5mM EDTA, at a concentration of 0.2g wet weight cells/ml and lysed with three passages through a French pressure cell at 20,000 psi. The lysate was clarified by centrifugation at 20,000xg for 30 minutes, and the insoluble fraction discarded. The protein concentration of the lysate supernatant was diluted to 5 mg/ml with lysis buffer and applied to a QAE-Toyopearl 550C anion-exchange column (Toso-Haas). The column was washed extensively with lysis buffer, and the bound fraction containing the fusion protein was then eluted with lysis buffer containing 100mM NaCl. This eluent was adjusted to 2M NaCl and applied to a phenyl-Toyopearl 650S column (Toso-Haas). The bound fusion protein was eluted with lysis buffer containing 0.5M NaCl.

Enteropeptidase cleavages. The *trx*A/IL-11 fusion protein purified as described above was dialyzed against lysis buffer to reduce ionic strength prior to enteropeptidase cleavage. The fusion protein was then combined with bovine enteropeptidase (Biozyme, EK-3 grade, specific activity = 1.8×10^5 units/mg) in 25mM Hepes, pH 8.0/5mM EDTA, at an enzyme:substrate ratio of 1:2000 (w/w) and incubated at 37°C for 15 hours. The EDTA was added to the reaction to eliminate minor secondary proteolysis of the IL-11 product. The reaction was terminated by addition of *p*-aminobenzamidine to 5mM, and the products were analyzed on a 10% tricine gel[56].

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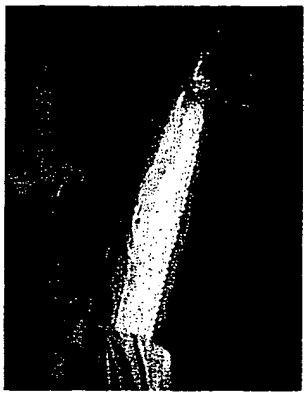

The Cover. *The first field trials of an insect-resistant transgenic corn yield a bounty of encouraging data.* ***See p. 194.*** *Photo © 1992, Comstock, Inc.*

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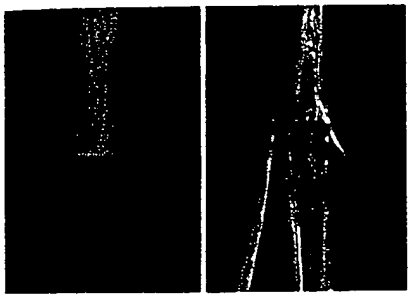

Field trial results:*The stalks of insect-resistant transgenic maize plants are intact (left), while those of nontransgenic plants show extensive insect tunneling damage (right).* *See p. 194.*

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